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Corrosion inhibition by aerobic biofilms on SAE 1018 steel

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Abstract Carbon steel (SAE 1018) samples were exposed to complex liquid media containing either the aerobic bacterium *Pseudomonas fragi* or the facultative anaerobe *Escherichia coli* DH5 α . Compared to sterile controls, mass loss was consistently 2- to 10-fold lower in the presence of these bacteria which produce a protective biofilm. Increasing the temperature from 23 °C to 30 °C resulted in a 2- to 5-fold decrease in corrosion inhibition with *P. fragi* whereas the same shift in temperature resulted in a 2-fold increase in corrosion inhibition with *E. coli* DH5 α . Corrosion observed with non-biofilm-forming *Streptomyces lividans* TK24 was similar to that observed in sterile media. A dead biofilm, generated *in situ* by adding kanamycin to an established biofilm, did not protect the metal (corrosion rates were comparable to those in the sterile control), and mass loss in cell-free, spent Luria-Bertani (LB) medium was similar to that in sterile medium. Confocal laser scanning microscopy analysis confirmed the presence of a biofilm consisting of live and dead cells embedded in a sparse glycocalyx matrix. Mass-loss measurements were consistent with microscopic observations of the metal surface after 2 weeks of exposure, indicating that uniform corrosion occurred. The biofilm was also able to withstand mild agitation (60 rpm), provided that sufficient time was given for its development.

Introduction

It has been estimated that the yearly corrosion damage costs are currently equivalent to 4.2% of the U.S. gross

national product (Martinez 1993). These costs could be greatly reduced by better and wider use of corrosion protection techniques. Traditional methods of corrosion protection involve the use of organic coatings to protect metal surfaces through barrier and passivation mechanisms (Funke 1986). However, these coatings are not permanent and the cost of applying organic coatings on corroding components in use is extremely prohibitive. Applying coatings before the components are introduced into service involves excessive costs because they are susceptible to abrasions and other forms of mechanically induced damage. Thus, a coating that can be easily applied and maintained on corroding parts and is cost-effective is an attractive alternative to the prevention methods currently in use. Since bacteria can coat metals with a regenerative biofilm (Pedersen and Hermansson 1989), it is becoming evident that they may be used as a means of preventing corrosion (Pedersen and Hermansson 1991).

Biofilms are adherent microbial populations that are trapped in an exopolysaccharide matrix (Hoyle et al. 1990). Most bacteria can attach to surfaces efficiently when they are viable and in a metabolically active state (Costerton and Lewandowski 1995). The degree and efficiency of adhesion depend on the extent of exopolysaccharide production by the bacteria. Both *P. fragi* (Parolis et al. 1991) and *E. coli* DH5 α (Huang et al. 1994) are known to produce glycocalyx and form biofilms. Glycocalyx helps the bacteria adhere to surfaces and that of *P. fragi* is composed of substituted glucose-mannose-glucose residues (Parolis et al. 1991). Glycocalyx also acts as a diffusional barrier to cationic molecules (Hoyle et al. 1990).

Pseudomonads (and other aerobic bacteria) have been shown to both increase and decrease the rate of metal corrosion through polymer production during biofilm formation (Black et al. 1988; Nivens et al. 1986). Geesey and Jang (1989) suggest that some biopolymers have differing metal-ion binding properties that can enhance corrosion. *Pseudomonas* sp. 200 is known to accelerate corrosion with increasing growth rate (Obuekwe

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et al. 1987). Jack et al. (1992) found that monocultures of an aerobic *Bacillus* sp. induced greater corrosion initially, but the rate of this corrosion decreased to that of a sterile control after 17 days. This suggests that bacterial metabolism might play a role in microbially influenced corrosion. Aerobic bacteria can create localized anaerobic niches in a biofilm, which allows sulfate-reducing bacteria to colonize and increase corrosion (Hamilton 1985). Under certain favorable conditions, pseudomonads have also been able to prevent corrosion (Pedersen and Hermansson 1989, 1991). Thomas et al. (1988) suggest that the presence of a biofilm may act as a diffusional barrier to the corrosive substances and prevent them from reaching the metal surface.

In this study, protective biofilms for corrosion prevention were generated with stationary and mildly shaken cultures of *P. fragi* and *E. coli* DH5 α . The main objectives of this study were to investigate the mechanism responsible for corrosion inhibition and to assess the potential of a biopolymer film for corrosion inhibition. This is the first report of corrosion inhibition with these strains. The effect of increasing temperature (i.e., changing the biofilm's metabolic state) on corrosion inhibition was determined, and antibiotic-resistant bacteria were created by genetic manipulation to avoid the influence of contamination as has been described in other reports (Hernandez et al. 1994). The protective ability of an intact non-viable biofilm was evaluated by adding kanamycin to kill biofilm bacteria *in situ*, and non-biofilm-forming *S. lividans* was used to assess the role of metabolites in corrosion inhibition. The ability of a fully developed biofilm to inhibit corrosion with mild agitation was also probed.

Materials and methods

Bacterial strains, medium, growth conditions, and specific growth rate

P. fragi was obtained from the American Type Culture Collection (ATCC no. 4973). A stable kanamycin-resistant derivative of *P. fragi* (*P. fragi* K) was generated by inserting a 3.2-kb *Tn5* mini-transposon (DeLorenzo et al. 1990) from *E. coli* S17-1 λ pir(pCNB4) (DeLorenzo et al. 1993) into the *Pseudomonas* chromosome using a modified biparental mating conjugation method (Fukuda 1994). A tetracycline-resistant derivative was also generated by electroporating *P. fragi* with the plasmid pKMY319 (Yen 1991) using a Gene Pulser (BioRad, Hercules, Calif.) at 1.1 kV and 25 μ F. *E. coli* DH5 α (pKMY319) (Yen 1991), *E. coli* S17-1 λ pir(pCNB4) (DeLorenzo et al. 1990), and *S. lividans* TK24 (Wang et al. 1990) were obtained from Prof. J. D. Lipscomb, Prof. Victor DeLorenzo, and Prof. D. L. Crawford respectively.

All strains were cultivated with multiple SAE 1018 metal coupons, prepared as described below, in 35 ml Luria-Bertani (LB) medium (Maniatis et al. 1982) supplemented with suitable antibiotics (Table 1) in 250-ml conical flasks without shaking in dark cabinets. The growth temperature was 23 °C or 30 °C. All strains were streaked from a -85 °C glycerol stock onto LB agar plates with appropriate antibiotics. A single colony was then picked and used to inoculate 10 ml of LB medium with suitable antibiotics and grown overnight at 23 °C or 30 °C, 250 rpm (New Brunswick Scientific, Edison N. J., series 25 shaker). A 0.1% inoculum from this overnight flask was used as the seed for corrosion experiments.

The growth medium was replenished every 7 days except for the short-term experiments (less than 7 days) and in the experiments with killed cells (the medium was not replaced after the cells were killed).

The specific growth rate was determined by monitoring cell growth (Drew 1981) using a Spectronic 20D spectrophotometer (Milton Roy, Rochester, N. Y.). Overnight cultures were grown as described above, diluted 50-fold and grown to a maximum absorbance (600 nm) of 0.80 to determine the specific growth rate.

Metal coupon preparation

SAE 1018 steel coupons, weighing 5.1 gr and having a thickness of 3 mm, were cut from a 25.4-mm-diameter rod and polished with 240 grit polishing paper (Buehler, Lake Bluff, Ill.). The polished samples were rinsed with distilled water, weighed, cleaned ultrasonically (Fisher Scientific Co. Pittsburgh, Pa., FS-3 series) in ethanol for 1 min, degreased with acetone, air-dried, and stored under vacuum. Samples were autoclaved at 121 °C for 20 min under a gravity cycle in dry conical flasks before the addition of growth medium and inoculation. All cultures were generated from stocks stored at -85 °C in 20% (v/v) glycerol.

Mass loss determination

The mass loss observed (mg/cm²) was used as an indicator of the extent of corrosion. At each indicated time, one of the multiple flasks containing a coupon was harvested, and the coupon was carefully removed from the growth medium. The biofilm was removed by repeated washing with distilled water and wiping the surface. The coupons were immediately dried and cleaned by scrubbing with synthetic rubber under warm running water.

Corrosion with a dead-cell biofilm

P. fragi (pKMY319) and *E. coli* DH5 α (pKMY319) were grown for 1–3 weeks, then the cells were killed by adding 200 μ g/ml kanamycin (Fisher Scientific Co. Pittsburgh, Pa.). Coupons were removed 1 week after addition of kanamycin and weighed to determine the mass loss. A section of the biofilm was scraped from the coupon and plated on LB agar plates to confirm that the biofilm bacteria had been killed by adding the antibiotic. Corrosion results were compared to controls consisting of coupons in sterile LB medium and LB medium with *P. fragi* that was not killed. The growth medium was not replenished in any of the flasks after addition of antibiotic, to avoid disruption of the biofilm.

Generation of spent medium

After *P. fragi* (pKMY319) had been grown for 5 days in the presence of a metal coupon without agitation to an absorbance (600 nm) of 2.95, the coupon was removed and the culture was sterilized by centrifuging at 7000 *g* for 10 min and passing it through a sterile 0.2 μ m filter (Gelman Sciences, Ann Arbor, Mich.). Kanamycin (200 μ g/ml) was added to the filtrate, and the solution was added to a sterile flask containing a metal coupon.

Biofilm resilience

A bacterial biofilm was allowed to form on metal coupons by growing the strains in LB medium with appropriate antibiotics in conical flasks without agitation. After 1 week, the medium was replenished and the flasks transferred to a series 25 rotary shaker at 23 °C and 60 rpm. The coupons were removed after 1 week and the mass loss determined.

Table 1 Specific growth rates and antibiotic resistance of the bacteria

Strain	Antibiotic resistance ($\mu\text{g/ml}$)	Specific growth rate at 23 °C (h^{-1})	Specific growth rate at 30 °C (h^{-1})	Source
<i>P. fragi</i>	None	0.55 ± 0.03	0.64 ± 0.02	ATCC no. 4973
<i>P. fragi</i> K	Kanamycin (100)	0.53 ± 0.02	0.64 ± 0.01	This study
<i>P. fragi</i> (pKMY319)	Tetracycline (50)	0.29 ± 0.04	0.43 ± 0.03	This study
<i>E. coli</i> DH5 α (pKMY319)	Tetracycline (25)	0.26 ± 0.02	0.34 ± 0.03	J.D. Lipscomb
<i>S. lividans</i> TK24	None	Not determined	Not determined	D.L. Crawford

Confocal laser scanning microscopy (CLSM) and metallography

Metal coupons with biofilm on the surface were removed from conical flasks and immersed once in 0.85% NaCl to remove bulk supernatant cells. The coupons were then stained for 30 min using the live/dead *BacLight* bacteria viability assay kit (Molecular Probes Inc., Eugene Ore.). The stained coupons were transferred to the stage of a confocal laser scanning (Kr/Ar) microscope (MRC600, Bio-Rad, Hercules, Calif.) equipped with a 60 \times , 1.4-NA oil-immersion lens, and a coverslip was added to the coupon, which was found to change the biofilm thickness and structure insignificantly. The sample was excited at 488 nm, and fluorescent light was imaged using the K1/K2 filter-block combination. Polysaccharide was stained with calcofluor (Sigma, St. Louis, Mo.) and visualized using the K1/K2 filter-block combination. Optical micrographs of sample coupons, after cleaning, were obtained with a Nikon Epiphot (Nikon, Garden City, N.Y.) using a 20 \times , 0.6-NA objective lens.

Results

Corrosion inhibition with *P. fragi* and *E. coli*

The growth rates of all strains used in this study, in LB medium at 23 °C and 30 °C, and their antibiotic resistances are given in Table 1. Mass loss in LB medium with either *P. fragi* or *E. coli* was examined for 4–6 weeks in stationary batch cultures at 23 °C and 30 °C (Fig. 1a,b). The mass loss observed was normalized relative to the total surface area (12.4 cm²) of the metal coupon. These results demonstrate that the coupons immersed in suspensions of biofilm-forming bacteria (all but *S. lividans* TK24) showed a 2- to 10-fold decrease in mass loss after 4 weeks compared to the coupons exposed to sterile LB medium. Figure 1a,b also indicates that 15%–40% of the total mass lost in 4 weeks in the presence of both bacteria occurred during the first week of exposure. Note that 0.03 ± 0.01 mg/cm² of this initial mass loss was a result of autoclaving the metal coupons.

For the pseudomonads, the mass loss was similar for wild-type *P. fragi*, *P. fragi* (pKMY319), and *P. fragi* K, indicating that the addition of antibiotic resistance did not affect corrosion inhibition. However, the degree of protection of the *Pseudomonas* strains was dependent on the cultivation temperature: the protective effect was more pronounced at 23 °C than at 30 °C. Figure 1b shows that the mass loss at 30 °C for the pseudomonads is 2- to 5-fold more than that observed at 23 °C. However, it was still 3-fold lower than the mass loss observed in sterile LB medium.

This protection afforded by the biofilm was corroborated by optical micrographs (Fig. 2a–c). Figure 2a,b

shows the typical surface appearance of a polished test sample prior to exposure and after 2 weeks of exposure to sterile LB medium respectively; the grooves created by polishing are no longer visible on the surface of the sterile control. Figure 2c indicates that the surface of coupons exposed to *P. fragi* K for 2 weeks in LB medium at 23 °C still contains the polishing grooves. These grooves were visible on the surface of the metal coupon even after 8 weeks of exposure in the presence of wild-type *P. fragi*.

The mass loss with *E. coli* DH5 α (pKMY319) was also investigated, and Fig. 1a shows that this organism

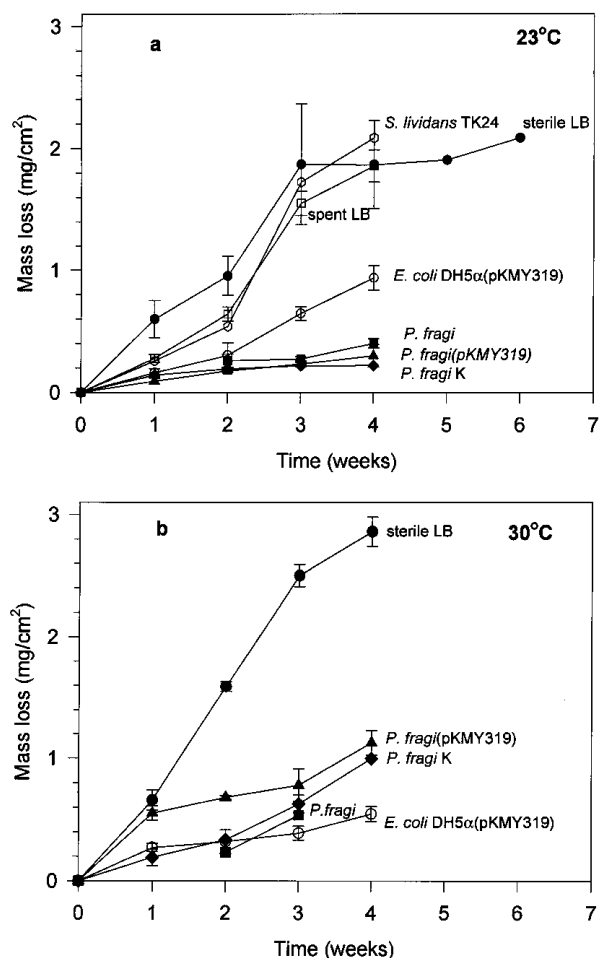


Fig. 1a, b Corrosion (mg/cm²) of SAE1018 steel coupons in Luria-Bertani (LB) medium with *Pseudomonas fragi* and *Escherichia coli* at 23 °C (a) and 30 °C (b). Data represent the average of three independent experiments (standard deviation error bars shown)

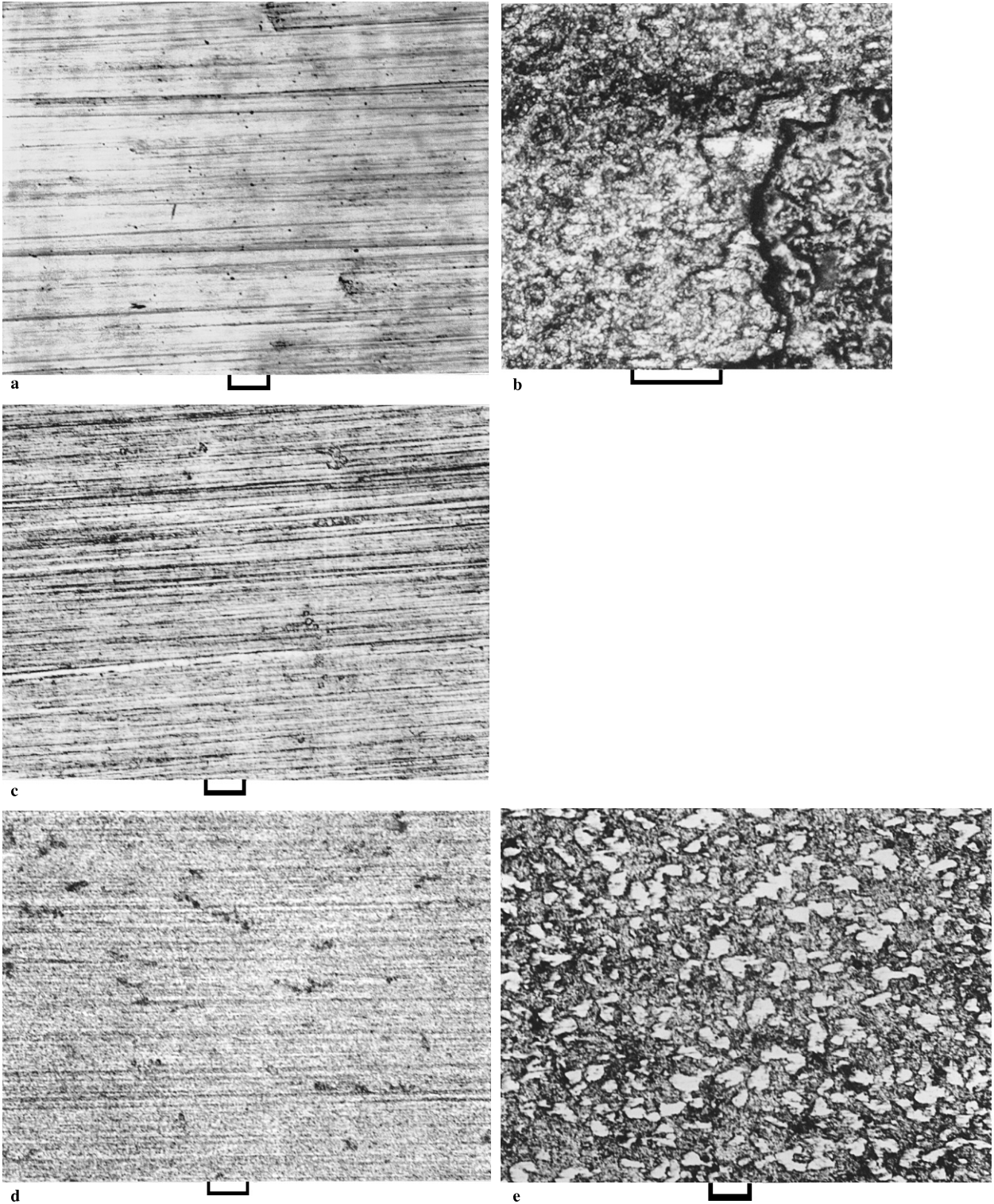


Fig. 2a–e Optical micrographs of SAE1018 steel (scale bar 50 μm in length). **a** Untreated sample, polished to 240 grit; **b** sample exposed to sterile LB medium for 2 weeks at 23 $^{\circ}\text{C}$; **c** sample exposed to LB medium with *P. fragi* K for 2 weeks at 23 $^{\circ}\text{C}$; **d** sample exposed to LB

medium with *E. coli* DH5 α (pKMY319) for 2 weeks at 23 $^{\circ}\text{C}$; **e** sample exposed to LB medium with *S. lividans* TK24 for 2 weeks at 23 $^{\circ}\text{C}$

also reduced the extent of mass loss from metal coupons as compared to a sterile control. Unlike, *P. fragi* K, *E. coli* DH5 α (pKMY319) protected better at 30 °C than at 23 °C. At 23 °C, the mass loss was nearly 3-fold more than that of coupons exposed to *P. fragi* but was still only half that of a sterile control. At 30 °C, the mass loss observed with *E. coli* DH5 α (pKMY319) was significantly lower than that of *P. fragi*(pKMY319) (Fig. 1b), and a sterile LB control exhibited nearly 12-fold more mass loss. The optical micrograph in Fig. 2d shows that grooves on the coupon surface with *E. coli* DH5 α for 2 weeks at 23 °C are less visible than those on a coupon surface with *P. fragi* (Fig. 2c); hence, the extent of corrosion is greater with *E. coli* at 23 °C.

Corrosion inhibition with a dead biofilm

Although *P. fragi* and *E. coli* inhibited corrosion, it was important to determine whether viable cells are required for this protection or if a layer of dead cells and glyco-calyx could inhibit corrosion to a similar degree. To kill the biofilm, kanamycin (200 μ g/ml) was added to the growth medium after 1–3 weeks of growth, *P. fragi* in suspension cultures was completely killed by 50 μ g/ml kanamycin; yet, since sessile bacteria exhibit an increased resistance to antibiotics (Hoyle et al. 1990; Suci et al. 1994), a 4-fold excess of kanamycin was used in our experiments to produce a dead biofilm. The biofilm bacteria were completely killed within 48 h of the addition of kanamycin, as shown by the absence of growth on LB agar plates. Figure 3a, b indicates that the formation of a dead biofilm *in situ* resulted in a 2- to 4-fold increase in mass loss after 1 week with both bacteria. The corrosion rate observed with a dead biofilm was comparable to that observed with sterile controls (Fig. 3a,b).

Corrosion inhibition with spent medium

Since live cells inhibit corrosion more than dead cells, it was necessary to investigate whether any cellular metabolites produced during the exponential growth or stationary phases of the bacterium were responsible for the corrosion inhibition (rather than the biofilm). Figure 1a shows that the mass loss obtained with cell-free, spent LB medium (obtained from a 5-day-old *P. fragi*/pKMY319 culture that reached an OD₆₀₀ of 6.45) was comparable to that obtained with fresh, sterile LB medium. This indicates that the metabolic products generated by the cell during its exponential growth and stationary phases are not significantly involved in the mechanism responsible for the reduction of corrosion. Pedersen and Hermanson (1991) also observed that cell-free spent medium could not sustain any fresh bacterial growth and did not inhibit corrosion. Since the spent LB medium was filter-sterilized rather than autoclaved, metabolic products were not degraded during the sterilization.

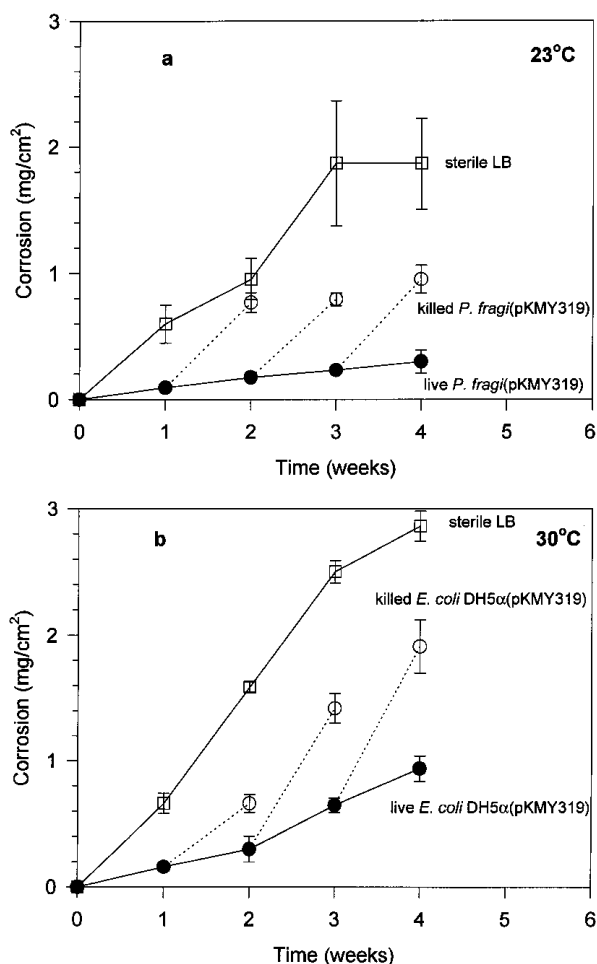


Fig. 3a, b Corrosion (mg/cm²) of SAE1018 steel in LB with a live and dead biofilm. The biofilm was killed *in situ* by the addition of 200 μ g/ml kanamycin and not disturbed further. Data represent the average of three independent experiments

Corrosion inhibition with non-exopolysaccharide-producing *Streptomyces lividans* TK24

To corroborate that the corrosion inhibition that was observed with *P. fragi* and *E. coli* was due to the presence of a bacterial biofilm and not to other extracellular factors, *S. lividans* TK24 (a sporulating soil bacterium, which is not known to form a biofilm or produce exopolysaccharides like glyco-calyx) was cultivated with coupons. Figure 1a demonstrates that the mass loss observed with *S. lividans* TK24 after 2 weeks was significantly higher than that observed with *P. fragi* and *E. coli*, but slightly lower than that observed in sterile LB medium (Fig. 1a). Thus, a non-sliming, non-biofilm-forming bacterium could not prevent corrosion to the same extent as the biofilm-forming *P. fragi* and *E. coli*. It should be noted that *S. lividans* grew in clumps in LB medium, and the culture supernatant did not attain the same absorbance (600 nm) as *P. fragi* or *E. coli* DH5 α . CLSM analysis indicated the presence of clumps of

S. lividans TK24 on the surface of the metal but not to the same extent as *P. fragi* or *E. coli*. Figure 2e shows an optical micrograph of a SAE 1018 steel coupon exposed to LB medium and *S. lividans* TK24 for 2 weeks at 23 °C. The surface is completely corroded and the scratch marks are no longer visible. It appears that virtually no protection was offered, and the micrograph is comparable to those obtained without any bacteria (Fig. 2b).

Biofilm resilience

The ability of the biofilm to impart corrosion resistance to metal under conditions where fluid flows is significant for many industrial applications. Hence, flasks containing coupons were inoculated with bacteria and the impact of shaking was determined (Table 2). When the flasks were incubated on a rotary shaker for 1 week without an initial stationary phase, the mass loss observed was quite high. Virtually no difference in mass loss was observed between the sterile LB medium control and the flasks with the bacteria (the mass loss was greater in the flask containing *E. coli* compared to the sterile LB medium flask). When flasks were shaken for 2 weeks without an initial stationary period, maximum mass loss was observed. However, when the flasks were initially incubated in a stationary mode at 23 °C for 1 week before being shifted to a rotary shaker, a significant difference in mass loss was observed, with the flask containing *P. fragi* K showing nearly half the mass loss observed with sterile LB medium. Again, the flask with *E. coli* exhibited more mass loss than the other two flasks. It is expected that shaking and agitation will cause an increase in the mass loss observed. These experiments confirm the protective ability of *P. fragi* under quiescent conditions as well as under mild agitation (60 rpm). *E. coli* DH5 α (pKMY319) offered virtually no protection when subjected to shaking while it offers significant protection under quiescent conditions.

Discussion

At 23 °C and 30 °C, in a rich medium, *P. fragi* and *E. coli* protect carbon steel coupons by inhibiting corrosion

2- to 10-fold over 4 weeks of exposure (Fig. 1a,b). These results compare well to those of Pedersen and Hermansson (1989), who reported an 8-fold reduction in mass loss with *Pseudomonas* S9 and *Serratia marscens* (a facultative anaerobe) after 19 days of exposure.

In this study, *P. fragi* was more efficient than *E. coli* DH5 α (pKMY319) in inhibiting corrosion at 23 °C, whereas *E. coli* was more efficient at 30 °C. Pedersen and Hermansson (1989) reported that *Pseudomonas* S9 protected metal better than *S. marscens*. The specific growth rate does not apparently determine the degree of protection since *P. fragi* grows faster than *E. coli* at both temperatures, and *P. fragi* K and *P. fragi* (pKMY319) protected metal to the same extent, although *P. fragi* (pKMY319) grows 33%–50% more slowly (Table 1, Fig. 1a,b).

To determine the protective ability of a dead (non-viable) biofilm, it is essential not to disturb the biofilm. Therefore, the cells were killed *in situ*, with minimal disruption of the biofilm, by the addition of the antibiotic kanamycin. Dead *P. fragi* and *E. coli* biofilms did not inhibit corrosion as effectively as a live biofilm and allowed corrosion to proceed at rates comparable to the sterile control once the film was killed (Fig. 3). These results agree with Hermansson and coworkers (Hernandez et al. 1994; Pedersen and Hermansson 1991), who used glutaraldehyde-fixed cells at 10⁹/ml to coat metal coupons and determined that metabolically inactive cells did not inhibit corrosion. These results, and our observations that cell-free, spent LB medium does not inhibit corrosion (Fig. 1a), suggest that living cells seem to be required for corrosion inhibition by *P. fragi* and *E. coli* DH5 α .

The biofilm resilience experiment has important practical considerations (Table 2). It demonstrates that the presence of bacteria alone is not sufficient to cause an inhibition of corrosion. When cells were transferred to a rotary shaker without an initial stationary phase, virtually no protection was observed, though comparable cell numbers were obtained in the supernatant (data not shown). When the flasks were transferred after a week-long stationary incubation period, the *P. fragi* K biofilm exhibited corrosion inhibition although it was nearly 5-fold less than that in stationary flasks (Table 2). This is expected, as agitation of the flasks (60 rpm) increased the mass loss (e.g., Table 2; 1 week mass loss of the shaken sterile control is 1.3 mg/cm² compared to 0.6 mg/cm² for a stationary flask). Surprisingly, the mass loss observed

Table 2 Effect of fluid agitation (60 rpm, 23 °C) on corrosion of SAE 1018 steel in the presence of a biofilm (where standard deviations are indicated, data represent the average of three independent experiments)

Conditions	Mass Loss (mg/cm ²)		
	Sterile LB	<i>P. fragi</i> K	<i>E. coli</i> DH5 α (pKMY319)
1 week shaking	1.3 \pm 0.1	1.4 \pm 0.3	4.5 \pm 0.2
1 week stationary	0.6 \pm 0.2	0.1 \pm 0.01	0.14
1 week stationary + 1 week shaking	1.7 \pm 0.2	0.9 \pm 0.1	4.4 \pm 0.8
2 weeks shaking	2.41	2.20	6.67
2 weeks stationary	0.96 \pm 0.16	0.19 \pm 0.01	0.16

with *E. coli* DH5 α (pKMY319) at 23 °C was higher than that observed in sterile LB medium. This could have been due to a corrosive cellular metabolite generated by *E. coli* DH5 α (pKMY319) which might cause the metal coupon to corrode more than in a quiescent mode of cultivation.

CLSM analysis indicates that a biofilm was present for all cultures except *S. lividans* TK24, and the biofilm consisted of cell clusters embedded in a sparse exopolysaccharide matrix (figure not shown). Live and dead cells (which stain green and red respectively) and void spaces were distributed throughout the biofilm. The presence of scratch marks on metal surfaces with biofilm-forming bacteria and the complete absence of scratch marks with sterile controls and *S. lividans*, along with the CLSM observations that *S. lividans* TK24 did not form a biofilm on the metal surface, clearly demonstrate that corrosion inhibition is due to the presence of the biofilm.

Oxygen levels near the surface of the biofilm may also influence the extent of corrosion observed. Hernandez et al. (1994) suggest that the protection of metal in a bacterial suspension may be due to a reduction in oxygen levels near the metal surface. However, Pedersen and Hermansson (1991) observed that lowering oxygen levels of sterile medium by 20% – 25% did not reduce corrosion levels to that observed with a *Pseudomonas* sp. S9 culture. Purging flasks containing metal coupons in sterile LB medium with nitrogen for 1 h once in 2 days resulted in a 10% decrease in corrosion levels after 1 week of exposure compared to sterile controls (data not shown). Therefore, complete removal of oxygen did not inhibit corrosion completely.

The overall findings suggest that an interaction between cellular metabolite(s) and live cells near the metal surface influences corrosion inhibition. It is, therefore, possible that corrosion inhibition is caused primarily by a live biofilm and its metabolism. The presence of a biofilm should cause localized pockets of oxygen depletion and result in a transient accumulation of cellular metabolites, all of which may contribute to the corrosion-inhibitory effect observed.

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References

- Black JP, Ford TE, Mitchell R (1988) Corrosion behavior of metal-binding exopolymers from iron and manganese-depositing bacteria. In: Corrosion 88, National Association of Corrosion Engineers, Houston, Texas
- Costerton WJ, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial biofilms. *Annu Rev Microbiol* 49: 711–745
- DeLorenzo V, Herrero M, Jakubzik U, Timmis KN (1990) Mini-*Tn5* transposon derivatives for insertion mutagenesis, promoter probing and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* 172: 6568–6572
- DeLorenzo V, Fernandez S, Herrero M, Jakubzik U, Timmis KN (1993) Engineering of alkyl- and haloaromatic- responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. *Gene* 130: 41–46
- Drew SW (1981) Liquid cultures. In: Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (eds) Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC, pp 151–153
- Fukuda M (1994) Degradation of recalcitrant synthetic chemical products: genetic engineering in pseudomonads. In: Murooka Y, Imanaka T (eds) Recombinant microbes for industrial and agricultural applications. Dekker, New York, pp 421–435
- Funke W (1986) Corrosion protection by organic coatings. In: Kendig MW, Leidheiser H Jr (eds) Symposium on corrosion protection by organic coatings. Electrochemical Society, Pennington, NJ, pp 1–10
- Geesey GG, Jang L (1989) Interactions between metal ions and capsular polymers. In: Beveridge TJ, Doyle RJ (eds) Metal ions and bacteria. Wiley, New York, pp 325–367
- Hamilton WA (1985) Sulphate-reducing bacteria and anaerobic corrosion. *Annu Rev Microbiol* 39: 195–217
- Hernandez G, Kucera V, Thierry D, Pedersen A, Hermansson M (1994) Corrosion inhibition of steel by bacteria. *Corrosion* 50: 603–608
- Hoyle BD, Jass J, Costerton JW (1990) The biofilm glycocalyx as a resistance factor. *J. Antimicrob Chemother* 26: 1–6
- Huang C-T, Peretti SW, Bryers JD (1994) Effects of medium carbon-to-nitrogen ratio on biofilm formation and plasmid stability. *Biotechnol Bioeng* 44: 329–336
- Jack RF, Ringelberg DB, White DC (1992) Differential corrosion rates of carbon steel by combinations of *Bacillus* sp., *Hafnia alvei* and *Desulfovibrio gigas* established by phospholipid analysis of electrode biofilm. *Corr. Sci* 33: 1843–1853
- Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Cold Spring Harbor, NY
- Martinez L (1993) The relationship of physical metallurgy and corrosion. *J Metals* 45: 21
- Nivens DE, Nichols PD, Henson JM, Geesey GG, White DC (1986) Reversible acceleration of the corrosion of AISI304 stainless steel exposed to seawater induced by growth of the marine bacterium *Vibrio natriegens*. *Corrosion* 42: 204–210
- Obuekwe CO, Westlake DWS, Plambeck JA (1987) Evidence that available energy is a limiting factor in the bacterial corrosion of mild steel by a *Pseudomonas* sp. *Can J Microbiol* 33: 272–275
- Parolis LAS, Parolis H, Dutton GGS, Wing PL, Skura BJ (1991) Structure of the glycocalyx polysaccharide of *Pseudomonas fragi* ATCC 4973. *Carbohydr Res* 216: 495–504
- Pedersen A, Hermansson M (1989) The effects on metal corrosion by *Serratia marcescens* and a *Pseudomonas* sp. *Biofouling* 1: 313–322
- Pedersen A, Hermansson M (1991) Inhibition of metal corrosion by bacteria. *Biofouling* 3: 1–11
- Suci PA, Mittleman, MW, Yu FP, Geesey GG (1994) Investigation of ciprofloxacin penetration into *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 38: 2125–2133
- Thomas CJ, Edyvean RGJ, Brook R (1988) Biologically enhanced corrosion fatigue. *Biofouling* 1: 65–77
- Wang Z, Bleakley BH, Crawford DL, Hertel G, Rafii F (1990) Cloning and expression of a lignin peroxidase gene from *Streptomyces viridosporus* in *Streptomyces lividans*. *J Biotechnol* 13: 131–144
- Yen K-M (1991) Construction of cloning cartridges for development of expression vectors in gram-negative bacteria. *J Bacteriol* 173: 5328–5335